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(54) Title: METHOD AND FORMULATION OF STIMULATING NITRIC OXIDE SYNTHESIS

(57) Abstract

A therapeutic mixture comprising a mixture of L-arginine and an agonist of nitric oxide synthase, namely nitroglycerin, is disclosed for the treatment of diseases related to vasoconstriction, wherein the vasoconstriction is relieved by stimulating the constitutive form of nitric oxide synthase (cNOS) to produce native nitric oxide (NO). The native NO having superior beneficial effect when compared to exogenous NO produced by an L-arginine independent pathway in terms of the ability to reduce clinical endpoints and mortality.

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1 # METHOD AND FORMULATION OF
2 ## STIMULATING NITRIC OXIDE SYNTHESIS

3 ## BACKGROUND OF THE INVENTION

4 This invention relates generally to a method of
5 treating hypertensive cardiocerebrorenovascular disease
6 as well as non-hypertensive cardiocerebrorenovascular
7 disease, and a unique formulation used in the treatment
8 of these diseases and their symptoms, wherein an
9 endogenous biological source of nitric oxide (L-arginine)
10 and a stimulator of Nitric Oxide Synthase (NOS),
11 particularly nitroglycerin, are mixed prior to
12 administration to form a mixture that is useful in the
13 treatment of nitroglycerin tolerance.

14 ## DESCRIPTION OF RELATED ART

15 For several decades nitroglycerin has been
16 administered to humans as a vasodilating agent in the
17 treatment of cardiovascular disease. Nitroglycerin or
18 glyceryl trinitrate is an organic nitrate ester which
19 when administered to a subject is converted biologically
20 to nitric oxide (NO) which is a pharmacologically active
21 metabolite. NO, for example, activates soluble guanylate
22 cyclase in vascular smooth muscle cells which in turn
23 increase cyclic guanosine monophosphate (cGMP) resulting
24 in vasorelaxation, (Waldman et al., 1987, *Cyclic GMP*
25 *synthesis and function*, Pharmacol. Rev. 39, 163.) and
26 ultimately leads to vasodilation and a reduction in blood
27 pressure. However, the effectiveness of nitroglycerin is
28 greatly diminished because the recipient of therapeutic
29 administration of nitroglycerin rapidly develops a
30 tolerance to the beneficial effects of nitroglycerin.
31 Therefore, onset of nitroglycerin tolerance significantly
32 limits the therapeutic value of nitroglycerin because
33 increased dosages have little or no effect on
34 vasorelaxation or vasodilation. (Bogaert, M., 1991,

1   *Clinical relevance of tolerance to nitrovasodilators*, J.
2   Cardiovas. Pharmacol. 17 (Suppl. 3), S313; and Unger, P.,
3   et al., 1991, *Tolerance to intravenous nitrates*, J.
4   Cardiovasc. Pharmacol. 17 (Suppl. 3), S300.) The precise
5   mechanism of nitroglycerin tolerance is unknown.
6   Theories explaining the tolerance include: the sulfhydryl
7   pools necessary for the direct biotransformation of
8   nitroglycerin into active nitric oxide are depleted by
9   excess nitroglycerin substrate. (Boesgaard, S., et al.,
10 1991, *Nitrate tolerance: effect of thiol supplementation*
11 *during prolonged nitroglycerin infusion in an in vivo rat*
12 *model*, J. Pharmacol. Exp. Ther. 258, 851); the activation
13 of vascular guanylate cyclase is diminished by
14 nitroglycerin (Henry P. J., et al., 1989, *S-Nitrosothiols*
15 *as vasodilators: Implications regarding tolerance to*
16 *nitric-oxide-containing vasodilators*, Br. J. Pharmacol.
17 98, 757); or that the rate of cGMP degradation may be
18 increased during tolerance to nitroglycerin (Axelsson, K.
19 L., et al., 1987, *Nitrate tolerance from a biochemical*
20 *point of view*, Drugs 33, 63).

21   Recently, nitric oxide has also been shown to be
22 formed enzymatically as a normal metabolite from arginine
23 in vascular endothelium to provide an important component
24 to the formation of endothelium-derived relaxing factor
25 (EDRF). Macrophages and neurons have also been shown to
26 produce nitric oxide in the body as a component of their
27 cell killing and/or cytostatic function.

28   More recently it has been established that a family
29 of enzymes called NOS form nitric oxide from L-arginine,
30 and the nitric oxide produced is responsible for the
31 endothelium dependent relaxation and activation of
32 soluble guanylate cyclase, nuerotransmission in the
33 central and peripheral nervous systems, and activated
34 macrophage cytotoxicity (Sessa, William C., 1994, *The*
35 *Nitric Oxide Synthase Family of Proteins*, Review, pp.
36 131-143,).

1   Nitric Oxide Synthase, occurs in many distinct
2 isoforms which include a constitutive form (cNOS) and an
3 inducible form (iNOS). The constitutive form is present
4 in normal endothelial cells, neurons and some other
5 tissues. Formation of nitric oxide by the constitutive
6 form in endothelial cells is thought to play an important
7 role in normal blood pressure regulation. The inducible
8 form of nitric oxide synthase has been found to be
9 present in activated macrophages and is induced in
10 vascular smooth muscle cells, for example, by various
11 cytokines and/or microbial products. It is thought that
12 in sepsis or cytokine-induced shock, overproduction of
13 nitric oxide by the inducible form of nitric oxide
14 synthase plays an important role in the observed life-
15 threatening hypotension.

16   As discussed above, the conversion of L-arginine
17 into nitric oxide is enzymatically catalyzed by NOS and
18 the resulting by- product is L-citrulline. Although it
19 was initially described in endothelium, as discussed
20 above, NOS activity has now been described in many cell
21 types. Brain, endothelium, and macrophage isoforms
22 appear to be products or different genes that have
23 approximately 50% amino acid identity. NOS in brain and
24 in endothelium have very similar properties, the major
25 differences being that brain NOS is cytosolic and the
26 endothelial enzyme is mainly a membrane-associated
27 protein.

28   Functionally, the constitutive form of Nitric Oxide
29 Synthase (cNOS), which is the predominant synthase
30 present in brain and endothelium, may be active under
31 basal conditions and can be further stimulated by
32 increases in intracellular calcium that occur in response
33 to receptor-mediated agonists or calcium ionophores.
34 cNOS appears to be the "physiological" form of the enzyme
35 and plays a role in a diverse group of biologic
36 processes. In vitro studies suggest that the activity of
37 nitric oxide synthase can be regulated in a negative

1 feedback manner by nitric oxide itself. In the
2 cardiocerebrorenovascular circulation, the primary target
3 for constitutively produced nitric oxide is soluble
4 guanylate cyclase located in vascular smooth muscle, the
5 myocardium (myocytes) and coronary vascular smooth
6 muscle.
7 In the presence of normal substrate, nitric oxide is
8 made preferentially by nitric oxide synthase. However,
9 in the absence of L-arginine, brain nitric oxide synthase
10 is thought to generate the free radicals superoxide and
11 hydrogen peroxide. This property of nitric oxide
12 synthase has potential major implications for
13 neurotoxicity and pathophysiological conditions such as
14 ischemia.
15 In contrast, to the constitutive form of the enzyme,
16 the inducible, calcium-independent form was initially
17 only described in macrophages. It is now known that
18 induction of nitric oxide synthase can occur in response
19 to appropriate stimuli in many other cell types. This
20 includes both cells that normally do not express a
21 constitutive form of nitric oxide synthase, such as
22 vascular smooth muscle cells, as well as cells such as
23 those of the myocardium (Levine B, et al., 1990, *Elevated*
24 *circulating levels of tumor necrosis factor in severe*
25 *chronic heart failure*. N Engl J med. 323:236-241.) that
26 express considerable levels of the constitutive isoform.
27 iNOS exhibits negligible activity under basal
28 conditions, but in response to factors such as
29 lipopolysaccharide and certain cytokines, expression
30 occurs over a period of hours. The induced form of the
31 enzyme produces much greater amounts of NO than the
32 constitutive form, and induced NOS appears to be the
33 "pathophysiological" form of the enzyme because high
34 concentrations of NO produced by iNOS can be toxic to
35 cells. Induction of iNOS can be inhibited by
36 glucocorticoids and some cytokines. Relatively little is
37 known about postranscriptional regulation of iNOS.

1 Cytotoxic effects of NO are probably largely independent
2 of guanylate cyclase and cyclic GMP formation.
3   Most of the research in the area has focused on
4 inhibitors of iNOS stimulation using various derivatives
5 of L-arginine. However little research has been done on
6 the stimulation of cNOS and its effect on nitroglycerin
7 tolerance. Nitroglycerin tolerance has continued to
8 frustrate the health care community because there is to
9 date no effective way to stimulate physiological NO
10 production above the tolerance or resistance floor of
11 nitroglycerin so as to maintain the beneficial effect of
12 the administration of nitroglycerin for prolonged
13 periods.
14   An effective method of treating hypertensive
15 cardiocerebrorenovascular diseases and symptoms as well
16 as non-hypertensive cardiocerebrorenovascular diseases
17 and symptoms so as to overcome the resistance-tolerance
18 floor of nitroglycerin is needed in the art.

19   SUMMARY OF THE INVENTION
20   The term "subject" is used herein to mean any
21 mammal, including humans, where nitric oxide formation
22 from arginine occurs. The methods herein for use on
23 subjects contemplate prophylactic use as well as curative
24 use in therapy of an existing condition. The term
25 "native NO" as used herein refers to the nitric oxide
26 that is produced through the biotransformation of L-
27 arginine or the L-arginine dependent pathway. The term
28 endpoints as used herein refers to clinical events
29 encountered in the course of treating cardiovascular
30 disease, up to and including death (mortality)
31   It is an object of this invention to treat
32 pharmacological tolerance to nitroglycerin.
33   It is another object of this invention to provide a
34 method of preventing, treating, arresting, or
35 ameliorating disease conditions which are benefitted by

1   the biotransformation of L-arginine into endogenous

2   nitric oxide or "native" nitric oxide.

3   It is another object of this invention is to provide

4   a formulation that has a combined arterial and

5   venodilatory effect.

6   It is another object of this invention to ameliorate

7   or avoid tachycardia and prevent or treat ischemia.

8   It is another object of this invention to premix L-

9   arginine and nitroglycerin to achieve a synergistic

10   effect to treat nitroglycerin tolerance by increasing or

11   maximizing the ability of nitroglycerin to produce

12   "native" nitric oxide, and reduce clinical endpoints to

13   include mortality.

14   It is another object of this invention to prevent

15   reperfusion injury in subjects who have had abrupt

16   restoration of blood flow.

17   It is another object of this invention to use the

18   combination or mixture formed to reduce the dosage

19   requirements of L-arginine and the corresponding

20   deleterious consequences of volume overload.

21   It is a further object of this invention to provide

22   a mixture of nitroglycerin and L-arginine for the

23   treatment of hypertension, hypertensive heart disease;

24   coronary heart disease, including angina, myocardial

25   infarction, and sudden death; and a wide range of

26   cardiovascular disease (heart failure, stroke, and

27   peripheral vascular diseases), and renovascular

28   ischemia/hypertension.

29   These and other objects of this invention are

30   provided by one or more of the embodiments provided

31   below.

32   In one embodiment of the invention, therapeutically

33   effective amounts of L-arginine and a cNOS agonist are

34   mixed together prior to administration to a subject.

35   In another embodiment of the invention,

36   therapeutically effective amounts of L-arginine and

1   nitroglycerin are combined at a physiologically
2   acceptable pH prior to administration.
3   In another embodiment a method for treating
4   hypertension in a subject by vasodilation or
5   vasorelaxation comprises: selecting a hypertensive
6   subject; administering to said subject an anti-
7   hypertensive formulation comprising a mixture of a venous
8   dilator; and an arterial dilator; obtaining periodic
9   blood pressure measurements of the subject; and;
10   continuing administration of the formulation until a
11   desirable blood pressure or therapeutic effect is
12   detected in the subject. A desirable blood pressure in a
13   hypertensive subject should ultimately be within the
14   following ranges: systolic preferably in the range of 95-
15   180 mmHg, more preferably in the range of 105-165 mmHg,
16   and even more preferably in the range of 120 to 140 mmHg;
17   and diastolic preferably in the range of 55-115 mmHg,
18   more preferably in the range of 65-100 mmHg, and even
19   more preferably in the range of 70 to 90 mmHg, and most
20   preferably 75-85 mmHg. Under no circumstances should the
21   systolic be permitted to go below 95 mmHg.
22   Another embodiment is a method for preventing or
23   treating cardiovascular disease in a non-hypertensive
24   subject by vasodilation or vasorelaxation comprising:
25   selecting a subject; administering to said subject a
26   formulation comprising a mixture of a venous dilator and
27   an arterial dilator wherein the venous dilator is a
28   combined non-endothelium and endothelium dependent source
29   of nitric oxide (i.e. nitroglycerin) and said arterial
30   dilator is an endothelium dependent source of nitric
31   oxide (L-arginine); obtaining periodic measurements of
32   vasorelaxation on the subject and; continuing
33   administration of the formulation until a desirable state
34   of vasorelaxation or desirable therapeutic effect is
35   detected on the subject. A desirable state of
36   vasorelaxation is for example a lowering of the systolic
37   by about 20 mmHg and a lowering of the diastolic by about

1   10 mmHg. Under no circumstances should the systolic be

2   lowered less than 95 mmHg.

3   Yet another embodiment is a method for treating

4   hypertension in a subject by vasodilation comprising:

5   selecting a hypertensive subject; administering to said

6   subject an anti-hypertensive formulation comprising a

7   mixture of L-arginine and nitroglycerin; obtaining

8   periodic blood pressure measurements on the subject; and;

9   continuing administration of the anti-hypertensive

10 formulation until a desirable blood pressure is detected

11 in the subject.

12   Yet another embodiment is a method for stimulating

13 cNOS in a subject which comprises: selecting a subject;

14 administering to said subject a formulation comprising a

15 mixture of L-arginine and nitroglycerin, so as to

16 maximize "native" NO production in order to treat

17 tolerance and reduce endpoints to include mortality.

18   BRIEF DESCRIPTION OF THE DRAWINGS

19   Fig. 1 is a schematic representation of the nitric

20 oxide production illustrating the proposed L-arginine

21 dependent and independent pathways.

22   Fig. 2 is a bar graph illustrating the cNOS

23 stimulating effect of combined administration of L-

24 arginine and nitroglycerin on rat aorta.

25   Fig. 3 is a bar graph illustrating the absence of

26 cNOS stimulating effect of combined administration of L-

27 arginine and SNP on rat aorta.

28   Fig. 4 is a human dose study which demonstrates the

29 absence of tachycardia during administration of the

30 herein described formulation.

31   DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

32   It has been discovered that combining L-arginine

33 with nitroglycerin prior to administration overcomes the

34 resistance or tolerance level normally established when

1   administering nitroglycerin alone. It is believed that
2   NOS may be stimulated by nitroglycerin and that premixing
3   with L-arginine has a synergistic beneficial effect that
4   may be due to a complex or coordinate formation between
5   nitroglycerin and L-arginine. Excess L-arginine provides
6   additional substrate for the stimulated nitric oxide
7   synthase which catalyzes the biotransformation of L-
8   arginine into nitric oxide.
9   Such stimulation of NOS in the presence of excess L-
10   arginine may be used to prevent, treat, arrest, or
11   ameliorate any disease or condition which may be
12   positively affected by NO production. Such conditions
13   include hypertensive cardiocerebrorenovascular diseases
14   and symptoms as well as non-hypertensive
15   cardiocerebrorenovascular diseases. The mixture is
16   particularly useful for subjects in need of native NO
17   production. Application of such a mixture is beneficial
18   for: (1) Chronic stable angina; (2) Unstable angina; (3)
19   Acute myocardial infarction; (4)   Hibernating
20   myocardium; (5) Stunned myocardium; (6) Limitation of
21   ventricular remodeling in post myocardial infarction and
22   subsequent risk   congestive heart failure; (7)
23   Prophylaxis of recurrent myocardial infarction; (8)
24   Prevention of sudden death following myocardial
     infarction; (9) Vasospastic angina; (10) Congestive heart
     failure-systolic-seen in association with 1-6 above; (11)
27   Congestive heart failure-diastolic-seen in association
28   with 1-10 above and 12-15 below; (12) Microvascular
29   angina seen in association with 1-11 above and 15 and 16
30   below; (13) Silent ischemia seen in association with 1-12
31   above and 15 and 16 below; (14) Reduction of ventricular
32   ectopic activity seen in association with 1-13 above and
33   15 below; (15) Any or all of the above 1- 4 states of
34   ischemic myocardium associated with hypertensive heart
35   disease and impaired coronary vasodilator reserve; (16)
36   control of blood pressure in the treatment of
37   hypertensive crisis, perioperative hypertension,

1   uncomplicated essential hypertension and secondary

2   hypertension; (17) Regression of left ventricular

3   hypertrophy seen in association with 15 and 16 above;

4   (18) Prevention and or regression of epicardial coronary

5   atherosclerosis seen in 1-17 above; (19) Prevention of

6   restenosis post angioplasty; (20) Prevention and/or

7   amelioration of free radical mediated reperfusion injury

8   in association with 1-19 above; (21) Use of the

9   combination in the prevention of myocardial injury during

10   cardioplegic arrest during coronary bypass or other open

11   heart surgery i.e. use of the combination as a

12   cardioplegic solution; (22) Post transplant

13   cardiomyopathy; (23) Renovascular ischemia; (24)

14   Cerebrovascular ischemia (Transient Ischemic Attack (TIA)

15   and stroke).

16   Fig. 1 is a schematic illustration showing the

17   proposed mechanism of action elicited by

18   nitrovasodilators on both a generator cell and a target

19   cell and their interrelationship. It appears that

20   nitroglycerin or glyceryl trinitrate's (GTN) mechanism

21   of action is both L-arginine dependent and L-arginine

22   independent and this implication has far reaching effects

23   regarding the development and treatment of nitroglycerin

24   tolerance and reducing clinical endpoints and mortality.

25   A type of generator cell is an endothelial cell, but may

26   also be an endocardial cell or a coronary endothelial

27   cell; and a corresponding type of target cell is a

28   vascular smooth muscle cell, but may also be a myocardial

29   cell (myocyte). Vascular smooth muscle cells are located

30   mainly in the veins, arteries, and coronary arteries.

31   The following discussion will focus on smooth muscle and

32   myocyte relaxation stimulated by nitrovasodilators

33   wherein the nitric oxide synthase is cNOS, the

34   constitutive form of nitric oxide synthase, the generator

35   cells are endothelial cells and the target cells are

36   vascular smooth muscle cells. This illustration is not

37   intended to imply any cellular relationship between the

various sites of action, but rather meant to illustrate
their functional relationship.

In Fig. 1 the production of NO may result from a
variety of sources and mechanisms which are discussed in
detail in Ignarro, (Louis J. PhD., 1991, *Pharmacology of
Endothelium-Derived Nitric Oxide and Nitrovasodilators*,
The Western Journal of Medicine, pp.51-62.) which is
incorporated herein in its entirety by reference. In the
L-arginine independent or non-endothelium dependent
pathway the activation of Guanylate Cyclase (GC) by
Nitric Oxide (NO) depends on the type of nitrovasodilator
used. Inorganic Nitrite (NO_2^-) is charged and only
limited amounts can permeate the cell, but intracellular
nitrite can be converted to NO. Lipophilic organic
nitrate esters (R-OH) are converted into NO by acidic
thiol (R-SH) facilitated reactions. S-Nitrosothiols (R-
SNO) are labile intermediates that decompose
spontaneously and produce NO. It is thought that one of
the mechanisms by which thiols potentiate the action of
nitroglycerin and reverse to some degree tolerance to
nitroglycerin is through the direct reaction between the
thiol (R-SH) and nitroglycerin (GTN) to form the labile
intermediate S-Nitrosothiol (R-SNO), which decompose as
described above (R-SH + GTN --> R-SNO is not shown in
Fig. 1). A nonenzymatic formation of exogenous NO is
thought to occur with thiol sources such as cysteine,
dithiothreitol, N-acetylcysteine, mercaptosuccinic acid,
thiosalicylic acid, and methylthiosalicylic acid.
Nitrates such as isosorbide dinitrate, and isosorbide 5'
mononitrate also can be used to produce NO since they are
simply commercially available intermediates to the known
L-arginine independent pathway. Nitroprusside ($(CN)_5$-
FeNO) forms NO upon breakdown and is not thiol dependent.
GTP is guanosine triphosphate; HONO is nitrous acid;
Meth. Blue is Methylene Blue; R-ONO is organic nitrite
esters; and R-SS-R represents a disulfide. In the L-
arginine independent pathway the glyceryl trinitrate

1   (GTN) reaction is represented by $R-ONO_2$ and are thought to

2   need a certain pool of thiols, such as a sulfhydryl

3   containing enzyme, to generate NO and it was formerly

4   thought that intracellular thiol deficiency results in

5   tolerance to the pharmacological actions of

6   nitroglycerin. This however does not account for the

7   tolerance because exogenous dose dependent thiols do not

8   result in reversal of nitroglycerin tolerance (Fung H.L.,

9   1988, *Journal of Pharmacology and experimental*

10 *Therapeutics.* 245:2,524-30.) but may exert beneficial

11 effect as independent donors of NO, versus facilitate

12 spontaneous release of nitric oxide. (Munzel T., M.D., et

13 al., 1994, *What Causes Nitroglycerin Tolerance?* Clinical

14 Cardiology. 20 No. 9:40-47.)

15   However, it is hypothesized for the first time here

16 that the tolerance to nitroglycerin may involve a

17 secondary pathway, or indeed, this "secondary pathway"

18 may be the primary pathway. This "secondary pathway" is

19 the L-arginine dependent pathway or endothelium dependent

20 pathway shown in Fig. 1. As seen in Fig. 1, the

21 generator cell is known to have several receptor mediated

22 agonists such as Endothelium B receptor (ET_B);

23 acetylcholine (Ach); substance P (SP), Histamine (H);

24 arginine vasopressin (AVP); bradykinin (BK); Adenosine

25 Triphosphate (ATP); Prostaglandin $F_{2\alpha}$ ($F_{2\alpha}$); Oxytocin,

26 (OT); and the calcium ionophore (A23187) which stimulate

27 the production of NOS. However, until now it has not

28 been speculated that nitroglycerin may serve the dual

29 role of agonist for NOS, and pro-drug for the sulfhydryl

30 mediated L-arginine independent pathway.

31   Previously it was thought that nitroglycerin had no

32 effect on the biotransformation of L-arginine into

33 "native" nitric oxide, but it is now believed that

34 nitroglycerin or a nitroglycerin complex or coordinate

35 (GTN complex in Fig. 1) with L-arginine has a stimulating

36 effect on cNOS. The mechanism is not well understood but

37 it appears the novel combination of nitroglycerin and L-

1 arginine prior to administration may have a heretofore
2 unexpected synergistic effect on cNOS stimulation which
3 may be due in part to a novel complex formulation that
4 serves as a delivery system of unprocessed nitroglycerin.
5 On the other hand the stimulation of cNOS may be a result
6 of cNOS having a unique receptor site for the complex or
7 nitroglycerin being in a state of disassociation
8 equilibrium with L-arginine. Administering the two in
9 combination also provides adequate substrate for cNOS
10 processing of L-arginine since the L-arginine will be
11 added in excess.

12   There appears to be some complex or coordinate
13 forming between L-arginine and nitroglycerin when the two
14 are mixed. This is shown in Table I, wherein the
15 coordinate was studied using a Bruker 300 MHz NMR. The
16 samples studied consisted of the following: Sample A, a
17 concentrated standard (100mg L-Arg in 0.5ml D_2O); Sample
18 B, a concentrated mixture (100mg L-Arg plus one tablet of
19 nitrostat in 0.5ml D_2O); Sample C, a diluted standard (1
20 drop of sample A in 1.0 ml D_2O); and Sample D, a diluted
21 mixture (13mg L-Arg plus 3 tablets of nitrostat in 1 ml
22 D_2O). These samples were compared and computer combined
23 to determine whether a complex had formed. The addition
24 of nitroglycerin to L-arginine resulted in a change in
25 the chemical shifts for L-arginine multiplet a $\partial 1.9$ and
26 triplet at $\partial 3.2$, the most readily studied signals. This
27 change is shown in Table I

TABLE I

Analysis of $\partial 3.2$ signal

	Signal Frequency	
sample C(Hz)	sample D(Hz)	change
979.032	980.119	1.087 Hz
972.107	973.281	1.174 Hz
965.272	966.364	1.092 Hz

Analysis of $\partial 1.9$ signal

	Signal Frequency	
sample C(Hz)	sample D(Hz)	change
582.392	584.513	2.121 Hz
575.108	577.287	2.179 Hz
573.365	575.607	2.242 Hz
567.231	569.348	2.117 Hz
565.698	568.118	2.420 Hz
559.425	561.673	2.248 Hz

The change in proton chemical shifts in L-arginine in the presence of nitroglycerin is a strong indicator that a complex of the substances is forming in solution to form an intermediate different from the two independent substances. This is further supported by the fact that the shift was not concentration dependent. Thus it may be fairly concluded that L-arginine and nitroglycerin do not act independently in solution but rather, are somehow involved in the formation of a complex which changes the chemical environment of the L-arginine protons and which can be detected using high resolution NMR spectroscopy. This may explain the unique beneficial NO delivery system which overcomes the resistance-tolerance threshold previously seen in the administration of nitroglycerin alone. However, the beneficial effect may merely result from the simultaneous administration of L-arginine and a cNOS stimulator.

Combining L-arginine and nitroglycerin may also result in a combined arterial and venous dilatory effect. Used alone nitroglycerin is principally a venodilator and causes rapid increase in heart beat due to its venous pooling, while L-arginine on the other hand when used alone is principally an arterial dilator. Therefore, combining the two results in balanced arterial and

1   venodilatory effect which counter balances the tendencies
2   of one or the other to produce tachycardia which is
3   adverse to ischemia in an evolving myocardial infarction.
4   This is suggested by preliminary data in dog stud⁻⁻s and
5   is most notable in the data shown in Table II. T: data
6   in Table II was generated by administering L-Arginine at
7   5 cc per minute wherein the L-arginine was at 10% w/v
8   (g/ml) and the nitroglycerin was administered at 3.38
9   μg/kg/minute by Intravenous (IV) administration over a
10 five minute period. The dog was a beagle that weighed
11 13.6 kg. When administered in combination, the relative
12 concentrations and dosages remained the same. BP is
13 Blood Pressure (systolic/diastolic in mmHg); MAP is Mean
14 Arterial Pressure (mmHg); CO is Cardiac Output
15 (liters/min.); TPVR is Total Peripheral Vascular
16 Resistance (dynes*sec./cm^3); ΔTPVR is the change in Total
17 Peripheral Vascular Resistance (%); and HR is Heart Rate
18 (bpm).

1   TABLE II - Canine Study

2 Agent	BP	MAP	CO	(TPVR)	HR	ΔTPVR
3 Before	130/75	93.3	1.44	(64.8)	105	
4 L-Arginine						31.6%
5 After	105/55	71.7	1.62	(44.3)	102	
6 Before	105/60	75.0	1.63	(46.0)	104	
7 Nitroglycerin						24.5%
8 After	70/40	50.0	1.44	(34.7)	105	
9 Before	105/60	75.0	1.56	(48.1)	102	
10 Nitroglycerin + L-Arginine						16.8%
11 After	70/40	50.0	1.60	(31.3)	98	

12   It can be seen from looking at The effect on CO or

13 Cardiac Output that after administration of the L-

14 arginine alone an increase in cardiac output is due to

15 the effect of L-arginine as principally an arterial

16 dilator; and the decrease in cardiac output seen with

17 nitroglycerin alone is principally due to a venous

18 dilatory effect; while the combination produces a

19 substantially balanced arterial and venous dilatory

20 effect (a change in cardiac output of only .04 (1.60 -

21 1.56)). Hence, the absence of a tendency towards

22 tachycardia (i.e. no evidence of baroreceptor reflex

23 activation).

24   Another mechanism of benefit from the combination

25 relates to the fact that used alone nitroglycerin is of

26 only minimal benefit in limiting reperfusion injury with

27 patients who have had recent heart attacks and abrupt

28 restoration of blood flow. The same thing is seen in

29 patients who are undergoing re-establishment of blood

30 flow after coronary bypass operations coming off the

31 bypass pump. This form of reperfusion injury is thought

32 to be mediated by free radical generation upon

33 reperfusion and preliminary data especially in cats shows

34 that L-arginine administered alone limits free radical

35 production. (Weyrich, A.S., PhD., et al., 1992, *The Role*

36 *of L-Arginine in Ameliorating Reperfusion Injury After*

37 *Myocardial Ischemia in the Cat.* Circulation. 86:279-288.)

1   Therefore, the combination would be likely to limit
2   reperfusion injury relative to nitroglycerin used alone.
3   Another benefit of the use of the combination
4   relative to each used alone relates to the fact that the
5   volunteer studies thus far with l-arginine alone reveal
6   it to be a weak vasodilator in terms of dosage
7   requirements. (600 cc/hr as reported by Nakaki T., et
8   al., 1990, *L-arginine Induced Hyportension.* The Lancet,
9   p. 696). Patients who have unstable coronary syndromes
10   and myocardial infarction with or without the
11   complication of congestive heart failure are prone to
12   volume overload with administration of IV fluids.
13   Therefore by combining nitroglycerin with L-arginine one
14   could limit remarkably the total L-arginine dosage
15   requirement and thereby the risk for developing
16   congestive heart failure. This might also be of
17   importance in patients who have compromised renal
18   function and are prone to acidosis and renal failure with
19   large volumes of L-arginine.
20   The principle combination to be employed will be a
21   mixture that involves therapuetic concentrations of L-
22   arginine and nitroglycerin in water. Any pharmaceutical
23   grade L-arginine will be sufficient and should be diluted
24   preferably to 2.5-60% w/v (g/ml), more preferably to 5-
25   45% w/v (g/ml), even more preferably between 7.5-30% w/v
26   (g/ml), even more preferably to 10-15% w/v (g/ml), and
27   most preferably 10% w/v (g/ml) L-arginine. The typical
28   doses anticipated will be 30 grams of L-arginine in
29   sterile water (Total Volume 300 cc). The L-arginine is
30   anticipated eventually to be approximately 10:1 to about
31   25:1 of the hydrochloride salt: L-arginine as a base, and
32   even more preferably 15:1 to about 20:1 hydrochloride
33   salt to base, and most preferably 15:1 hydrochloride salt
34   to base. In this example 28 to 29 grams will be the
35   hydrochloride salt and 1 to 2 grams of L-arginine will be
36   base. It is anticipated that the nitroglycerin to be
37   combined with L-arginine will have a concentration

1   dependent on the mass of the subject in kg and dosage
2   time preferably in the range of 0.1 μg/kg/minute to about
3   5 μg/kg/minute, more preferably in the range of 0.2
4   μg/kg/minute to about 4 μg/kg/minute, even more
5   preferably in the range of 0.5 μg/kg/minute to about 3
6   μg/kg/minute, even more preferably in the range of .75
7   μg/kg/minute to about 2 μg/kg/minute, and most preferably
8   about 1 μg/kg/minute. Therefore depending on the IV
9   volume, the administration time, and the weight of the
10   subject nitroglycerin will be added in an amount
11   sufficient to obtain the desired range (i.e. 1
12   μg/kg/minute). If a transdermal system is used the
13   delivery of nitroglycerin should preferably be between
14   0.2 mg/hr and 1 mg/hr, more preferably between 0.3 mg/hr
15   and 0.8 mg/hr, and even more preferably between 0.4mg/hr
16   and 0.6 mg/hr. It is anticipated that the package will
17   contain freeze dried L-arginine in a glass bottle to
18   which the nitroglycerin and sterile water would be added
19   in such as fashion as to have 30 grams of L-arginine and
20   1 to 960 milligrams of nitroglycerin all diluted to a
21   total volume with sterile water of 300 cc.
22   Alternatively, nitroglycerin, L-arginine, and water can
23   be added in sterilized glass bottles and adjusted to a
24   physiological pH. The pH on reconstitution in water
25   should preferably be in the range of approximately 5-8,
26   more preferably in the range of 6-7.5, even more
27   preferably in the range of 7 to 7.5, and even more
28   preferably approximately 7.4 which is physiologic in
29   order to avoid the present problem that is present in
30   those solutions that require the pH limitation of 5.6 to
31   avoid bacteriologic overgrowth on periods of prolong
32   standing when shipped in solution.
33   The dose of nitroglycerin might vary according to
34   future studies on the effect of the combination ratio on
35   heart rate. In addition even though the discussion
36   focuses on intravenous administration, buccal,
37   intracoronary, intramuscular, topical, intranasal,

1   rectal, sublingual, oral, subcutaneous, or patch
2   administration forms alone or in combination apply as
3   well. Because of their compatibility, the combination of
4   L-arginine and nitroglycerin in patch may be the most
5   common use as is the case presently for the use of
6   nitroglycerin alone in patch form. The feasibility of
7   patch technology is supported by solubility test of L-
8   arginine in Tridil™. Solubility test demonstrated the
9   following: without the addition of water, approximately
10   170 mg of L-arginine will dissolve in 1.0 ml of Tridil™
11   (5mg of nitroglycerin/ml); a clear colorless mixture was
12   obtained when 2500 mg of L-arginine hydrochloride, 1.0 ml
13   of Tridil™, and 2.8 ml of deionized water were combined
14   at 30°C with gentle swirling and then cooled to ambient
15   temperature (approximately 24°C); and a very thick, yet
16   pourable, slurry was obtained when 2500 mg of L-arginine,
17   1 ml of Tridil™, and only 0.5 ml of deionized water were
18   combined. These results suggest that L-arginine and
19   Tridil™ have a great degree of solubility compatibility
20   and therefore could easily be incorporated into the
21   current patch administration technology.
22   The following illustrate the above described
23   mechanism of action and treatment of
24   cardiocerebrorenovascular diseases:

                              Example 1

26   It was recently discovered that dogs treated to a
27   floor of nitroglycerin effect could be made further
28   responsive by the co-administration of nitroglycerin and
29   L-arginine in water in a manner similar to that commonly
30   seen clinically with the addition of sodium nitroprusside
31   (SNP) to nitroglycerin; however, when compared to SNP, L-
32   arginine combined with nitroglycerin had much more
33   favorable hemodynamic effects. Compared to SNP,
34   vascular resistance was reduced by 50%, cardiac output
35   doubled, and contractility increased. This led to the
36   hypothesis that the combination of L-arginine and
37   nitroglycerine was generating EDRF as opposed to SNP

1   which is known to produce nitric oxide in a direct
2   fashion.
3   Since there is still debate whether EDRF is
4   identical to nitric oxide it was hypothesized that EDRF
5   not being identical to NO would account for the
6   difference in hemodynamic effect. To account for the
7   extra EDRF it was hypothesized that nitroglycerin in
8   addition to being a pro-drug for nitric oxide was also an
9   agonist to cNOS activation and that L-arginine rate
10 limitations in the canine model could be explained by a
11 supply-demand mismatch in L-arginine uptake particularly
12 in disease state such as hypertension, hyperlipidemia,
13 arteriosclerosis involving the endothelial cell which is
14 thought to be an active transport process with potential
15 rate limitations which can possibly be overridden by
16 passive diffusion of L-arginine given in excess. Hence,
17 the rational for combining L-arginine with nitroglycerin
18 for the treatment of nitrate resistance and tolerance.
19 To test this hypothesis, the effects of exposing intact
20 rat aorta to nitroglycerin combined with L-arginine in
21 aqueous solution was studied and the results were
22 compared to the results obtained with SNP combined in an
23 aqueous solution with L-arginine. The effect of
24 combining L-arginine and nitroglycerin appear in Figure
25 2. The clinical preparations were as follows:
26 ANIMAL PREPARATION
27   Eight Sprague-Dawley rats were used in this
28 nitroglycerin study and two were used in the SNP study.
29 Following removal of the aorta from each rat the aorta
30 was cleaned and cut into 5 segments. The segments were
31 randomly distributed to minimize variation in baseline
32 values. Following this, the segments were incubated in
33 Earl's Salt solution at 37°C.
34 TREATMENT PROTOCOL
35   Nitroglycerin Group - one of the five segments
36 removed served as control to assess the integrity of the
37 endothelium (basal activity). The other four each

1   received 50 μmol of L-arginine. After 30 minutes 1ml of
2   IBMAX (50 μmol) was added to the 5 segments to prevent
3   any further cGMP degradation by phosphodiesterase (IBMAX
4   is isobutyl methyl xanthine). The 5 segments were
5   treated as follows: A - control-basal activity; B is L-
6   arginine group - 50 μmol L-arginine added to basal group;
7   C is the nitroglycerin group - 5 μmol nitroglycerin in
8   L-arginine 50 μmol; D is nitroglycerin + N^G-nitro-L-
9   arginine methyl ester (L-NAME a known inhibitor of NOS
10   function) group - 5 μmol nitroglycerin + .5m mol of L-
11   NAME and L-arginine 50 μmol; and E is the L-NAME group -
12   .5m mol of L-NAME and L-arginine at 50 μmol. After 50
13   minutes each of the segments were removed and placed in
14   500μ L of .1 NHCl. They were left for one hour at which
15   time they were removed and weighed.
16   <u>CYCLIC GMP ASSAY.</u>
17   For cGMP determination 400 μL of HCl solution
18   remaining after strips were removed and weighed were
19   transferred into gama flow tubes and cyclic GMP was
20   determined by radioimmunoassay.
21   <u>DATA INTERPRETATION</u>
22   A.   Control - Basal. This represents cGMP activity
23   at baseline that was generated by resting NO sources of
24   soluble guanylate cyclase activation, i.e. baseline.
25   B.   L-arginine Group. This represents cGMP
26   activity generated by L-arginine and EDRF (endogenous or
27   "native" NO production).
28   C.   Nitroglycerin Group. (L-arginine plus
29   nitroglycerin) The cGMP activity represents the sum of B
30   (L-arginine) plus nitroglycerin induction of cNOS and the
31   subsequent EDRF produced in addition to nitric oxide from
32   nitroglycerin by the L-arginine independent pathway (pro-
33   drug effects).
34   D.   L-NAME Group. L-arginine (L-arginine plus
35   nitroglycerin plus L-NAME). Represents cGMP activity from
36   nitroglycerin enzymatic conversion alone since L-NAME

1   used in excess inhibits NOS derived EDRF from all

2   sources.

3   E.   L-arginine + L-NAME - represents cGMP activity

4   due to non-nitric oxide sources activating soluble

5   guanylate cyclase activation and was subtracted from all

6   measurements to eliminate effects of non NO activation of

7   cGMP. (atrial natriuretic factor, etc.)

8   From this it is apparent that: Total NO from

9   nitroglycerin is C-B; NO from enzymatic degradation of

10 nitroglycerin to NO equals D-E; EDRF (NOS) stimulation

11 from nitroglycerin = (C-B) - (D-E)

12 SNP GROUP

13   A second group of two rats was examined, as above,

14 only in this group SNP was substituted in the treatment

15 protocol for nitroglycerin. These results are shown in

16 Fig. 3, A', B', and E' correspond exactly with A, B, and

17 E of Fig. 2. C' is equal to L-arginine at 50 μmol plus 1

18 μmol SNP and represents cGMP activity from L-arginine

19 stimulation of EDRF production plus any cNOS activation

20 by SNP plus NO from SNP by non-enzymatic conversion. It

21 does not appear that SNP requires any sulfhydryl group,

22 but rather that it forms NO and cyanide as a by-product

23 nonenzymatically. D' is SNP + L-NAME - represent cGMP

24 activity generated by non enzymatic conversion of SNP to

25 NO alone, i.e. exogenous or "non-native" NO. Total NO

26 from SNP = C'-B'; Total NO from SNP from non-enzymatic

27 conversion = D'-E'; EDRF from SNP by NOS activation =

28 (C'-B')-(D'-E').

29 RESULTS

30   Figures 2 and 3 summarizes these results with a bar

31 graph representative of the respective detected picomols

32 of cGMP/100 mg wet tissue. Although not shown in Fig. 2,

33 when nitroglycerin and L-NAME were combined in the

34 absence of L-arginine, similar results were obtained

35 regarding cGMP production. In both Figs. 2 and 3 the bar

36 labelled NOS is the amount of "native" NO produced which

1   is total NO minus the NO produced via the L-arginine
2   independent pathway.
3   Nitroglycerin resistance - tolerance has frustrated
4   cardiologists and pharmacologists since 1888. (Stewart
5   D.D., 1888, *Remarkable Tolerance to Nitroglycerin.*
6   *Philadelphia Polyclinic.* 172-5.) These results support
7   the hypothesis outlined in Fig. 1 and clarify the
8   mechanism of nitroglycerin tolerance. It is believed
9   that an additional nitroglycerin activation site is cNOS
10 in the endothelial cell. Under conditions leading to
11 tolerance the agonist effect of nitroglycerin on cNOS
12 induction leads to a depletion of L-arginine in the
13 endothelial cell secondary to rate limitations in active
14 L-arginine transport pump kinetics in Fig. 1. This
15 creates a supply demand mismatch situation at the
16 membrane uptake step and explains why arginine is rate
17 limiting in a canine model. This may also explain why
18 during administration of nitroglycerin a nitrate free
19 interval is required. It is believed that this is
20 necessary so that the endothelial cells can replete the
21 deficient L-arginine by active transport. By adding L-
22 arginine to nitroglycerin it is believed that EDRF can be
23 generated, and in the process a significant reduction in
24 clinical and mortality endpoints can be obtained relative
25 to using nitroglycerin alone or in combination with SNP
26 or other donors of exogenous NO.
27   The fact that veins are more sensitive to exogenous
28 NO (and most likely "native" NO also), compared to
29 arteries, explains why at low doses nitroglycerin is
30 principally a venous dilator compared to SNP which is a
31 balanced arterial venous dilator. It explains why at 37
32 micrograms/hr nitroglycerin becomes arterial ?cause at
33 this level all the EDRF potential is realize< and pro-
34 drug conversion of NO takes over as the last source of
35 nitric oxide generated by nitroglycerin. This last
36 source of NO generated from pro-drug conversion is

1   equivalent to NO from SNP and generates a similar

2   arterial effect.

3   It is possible that EDRF is not identical to NO and

4   is possibly the precursor (L-OH-NO half life of 3-50

5   seconds) for NO. This would seem to explain failed

6   attempts to substitute SNP for nitroglycerin in clinical

7   situations, such as unstable angina and acute myocardial

8   infarction (Flaherty, J.T., M.D., 1983, *Comparison of*

9   *Intravenous Nitroglycerin and Sodium Nitroprusside in*

10 *Acute Myocardial Infarction.* American Journal of

11 Medicine. 53-60.) since EDRF has better anti-ischemic

12 actions and since EDRF would not be produced using SNP,

13 SNP would not lead to the benefits in mortality

14 potentially realizable with nitroglycerin. Another

15 beneficial effect of EDRF produced by cNOS stimulation

16 with nitroglycerin may result from the ability of EDRF to

17 function as a free radical scavenger relative to

18 exogenous NO. (Zembowicz A., et al., 1991, *Nitric Oxide*

19 *and Another Potent Vasodilator are Formed from N^G-hyroxy-*

20 *L-arginine by Culture Endothelial Cells.* Pharmacology.

21 Proc. Natl. Acad. Sci. USA 88:11172-76.) In a

22 reperfusion injury a free radical scavenger (possibly

23 EDRF) is needed to absorb the free radicals which appear

24 to be what is happening with L-arginine and nitroglycerin

25 but not with SNP, a non-native source of NO. This can be

26 explained because one would not expect to see the

27 intermediate EDRF with SNP. Tolerance is established and

28 the beneficial effect of nitroglycerin is lost because

29 there is no longer any EDRF being produced or at least

30 until the rate limiting step is overcome by adding L-

31 arginine substrate. This serves an additional mechanism

32 of benefit from the combination or complex because it

33 relates to the fact that used alone nitroglycerin soon

34 loses its beneficial effect in limiting reperfusion

35 injury with patients who have had recent heart attacks

36 and abrupt restoration of blood flow. The same thing is

37 seen in patients who are undergoing re-establishment of

1   blood flow after coronary bypass operations coming off
2   the bypass pump. This form of reperfusion injury is
3   thought to be mediated by free radical generation of
4   reperfusion and preliminary data especially in cats show
5   that L-arginine administered alone also limits free
6   radical production. Therefore, the combination would be
7   likely to limit reperfusion injury relative to
8   nitroglycerin used alone.

9   These results indicate the formation of a new drug
10 by combining nitroglycerin with L-arginine in excess so
11 as to take advantage of passive diffusion override
12 mechanism of the endothelial cells membrane transport
13 pump as a treatment for nitroglycerin resistance-
14 tolerance. Such a formulation has applications which
15 include hypertension, hypertensive heart disease,
16 coronary heart disease (angina, myocardial infarction,
17 sudden death), cardiovascular diseases (congestive heart
18 failure, stroke, peripheral vascular disease),
19 cerebrovascular ischemia (TIA), and renovascular
20 ischemia.

21   Another potential utility of this complex is to
22 independently produce EDRF as seen here in rat aorta and
23 the canine results which will be of great value as a
24 treatment for tolerance of nitroglycerin without
25 additional toxicity or inconvenience in administration of
2   nitroglycerin presently used alone. The method of
27 administration would be unchanged.

28   It appears as though the L-arginine-nitroglycerin
29 mixture is stimulating cNOS selectively and is not
30 inducing iNOS. This is supported by the following:
31   1.   iNOS induction generally leads to irreversible
32   vascular collapse and death. The classic
33   example being endotoxic shock. This was not
34   seen in the present studies.
35   2.   iNOS induction is associated with a positive
36   feedback mechanism for increasing L-arginine
37   transport into the iNOS endothelial cell.

-25-

1   (Lind, D.S., M.D., 1993, *Endotoxin Stimulates*
2   *Arginine Transport in Pulmonary Artery*
3   *Endothelial Cells*. Surgery; 114;2; pp 199-
4   205). Supplementing L-arginine administration
5   would therefore only accelerate the tendency of
6   vascular collapse.

7   3.   In states wherein iNOS induction is not present
8   at baseline, the administration of
9   nitroglycerin, L-arginine, alone or combined,
10   does not lead to irreversible vascular
11   collapse. Both nitroglycerin alone or the
12   combination produce dose dependent hypotension
13   which is reversible upon the discontinuation of
14   the exposure to the respective drugs

15   Regarding paragraph 2 above, in states of iNOS
16 induction described above, it is believed that the
17 development of nitroglycerin tolerance may be an opposite
18 effect of nitroglycerin on the membrane pump, i.e a
19 negative feedback mechanism on the active L-arginine
20 membrane transport. This may be a factor which leads to
21 the development of tolerance.

22   Regarding paragraph 3 above, iNOS induction may be a
23 common feature of all vascular shock, including
24 hemorrhagic and cardiogenic shock. Advanced stages of
25 congestive heart failure with low output syndrome
26 (borderline cardiogenic shock) may likewise be associated
27 with cytokine production (Tumor Necrosis Factor) and
28 induction of iNOS. Care will need to be employed in the
29 future with administration of L-arginine in combination
30 with nitroglycerin in these states much in the same way
31 care is currently employed when administering
32 nitroglycerin alone when patients are hypotensive at
33 baseline.

34   An eight hour infusion in a normal human volunteer
35 has been performed using a wide range of nitroglycerin
36 concentrations ranging from 12.5 mg /250 cc total volume
37 through 100 mg/250 cc total volume 10% L-arginine and

1   found most importantly the absence of tachycardia
2   previously reported with either L-arginine or
3   nitroglycerin alone. In addition with 2 1/2 times the
4   currently approved dosages of L-arginine exposure (75 g
5   total) there was no evidence of metabolic acidosis from
6   the HCL present in the L-arginine formulation currently
7   approved. This study is summarized below.

8   Example 2
9   The following study is a normal human volunteer dose
10 ranging study for intravenous nitroglyce- in combined with
11 L-arginine. The objective of this stud s to examine
12 the combined administration of intravenous nitroglycerin
13 with L-arginine 10% (aqueous) for the following:
14   1.   Reflex tachycardia (baroreceptor reflex
15   activation).
16   2.   Hypotensive activity (therapeutic effect).
17   3.   Metabolic disturbances-metabolic acidosis.
18   4.   Electrocardiographic abnormalities with
19   prolonged infusion.
20   The patient studied in this dose ranging study was a
21 47 year old normotensive white male with no prior history
22 of illness or hospitalization and on no chronic
23 medications.
24   The materials utilized in this study consisted of
25 the following:
26   1.   Tridil brand of intravenous nitroglycerin (5mg
27 per cc).
28   2.   10% L-arginine in water (R-Gene™-KABI).
29   3.   Normal saline.
30   4.   5 x 150cc vacuum sealed sterile bottles.
31   5.   Two Ivac Pumps to include a 3 way stopcock for
32   alternating infusions of drug and saline.
33   6.   One Propac cardiac monitor.
34   7.   One Spacelabs 2000 24 hour blood pressure
35 monitor.

8. One Cardionostics Dural-Lite model #2011 holter recorder.

Patient preparation consisted of pretreatment with 40mg of Pepcid (famotidine-MERCK) and 50mg of benadryl the night before. 50mg of benadryl was repeated on the morning of the study. This was done for the purpose of blocking H_1 and H_2 receptors from any possible activation by L-arginine.

On the morning of the study a baseline EKG was obtained along with Serum Chemistries and Complete Blood Count (CBC). Following this the 24 hour holter monitor, ambulatory blood pressure monitor, and Propac were attached. The blood pressure monitor was calibrated against the Propac and a discrepancy of approximately 20 mmHg of systolic and 10 mmHg of diastolic blood pressure was observed in the left verses right arms respectively. Next, an IV was established in the left foot in the left saphenous vein with an 18 gauge angiocath. An initial maintenance infusion with saline was begun at KVO (keep vein open) rate. Following this six rapid dose response titrations were performed over the following 8 hours and are shown in Fig. 4 with ¼ (bottle #1), ½ (bottle #2), and full strength nitroglycerin in 10% L-arginine (bottle #3). This was followed by a full strength nitroglycerin infusion in water without L-arginine (bottle #4). Next an infusion of pure L-arginine 10% was administered without nitroglycerin in 10% L-arginine (bottle #5). Lastly an infusion consisting of double strength nitroglycerin in 10% L-arginine (bottle #6) was administered. Full strength nitroglycerin was defined as 50mg of nitroglycerin in a total volume of 250cc of L-arginine 10% in water or water alone (bottle #4).

With each infusion, the initial rate was 25cc per hour. Following this the infusion was doubled to 50cc per hour. This was increased by 50cc per hour every 5 to 10 minutes until a total infusion rate of 300cc per hour was achieved. During these infusions blood pressure and

1   heart rate data were recorded every 2 minutes by Propac
2   before increasing the rate of infusion as described
3   above. During bottle changes the infusion was changed to
4   normal saline at 100cc per hour. At the beginning of
5   each infusion an estimated 10cc of "dead space" was
6   eliminated from the infusate left over from the
7   previous bottle by running the first 10cc at a "wide
8   open" rate. Then the 25cc sequence was re-initiated as
9   previously described above.
10   Following the final infusion a repeat of Serum
11   Chemistries, CBC, and EKG were obtained.
12   For each infusion systolic and diastolic right arm
13   blood pressures were averaged. Heart rate was likewise
14   averaged. These averages were obtained by taking each
15   individual reading obtained every two minutes, totaling
16   them, and dividing the period in which the infusion
17   occurred (measurements in between infusions during bottle
18   changes not included).
19   The results are summarized in Fig. 4. In Fig. 4 SBP
20   means Systolic Blood Pressure, DBP means Diastolic Blood
21   Pressure and HR means Heart Rate. There does not appear
22   to be any evidence of reflex tachycardia with the ratio
23   of nitroglycerin to L-arginine used in Fig. 4. There was
24   a dose dependent blood pressure reduction along with a
25   trend toward dependency on nitroglycerin
26   concentration. There was no evidence of metabolic
27   acidosis developing secondary to L-arginine infused for a
28   prolonged period to the total dose of 75 grams
29   administered over 8 hours. There was no evidence of
30   arrhythmia. There was no evidence of
31   electrocardiographic abnormalities. Clearly, this
32   indicates that the administration of the combined
33   L-arginine/nitroglycerin does not have the adverse
34   consequences seen with either L-arginine or nitroglycerin
35   when administered alone.
36   The foregoing description of the invention is
37   illustrative of the preferred embodiments of the

1   invention currently contemplated by the inventor thereof.
2   However, it should be clear that the foregoing
3   description of the invention is not to be interpreted in
4   a limitative manner, there being several equivalent
5   systems and manners of performing the present invention.
6   For example, the L-arginine is contemplated to be derived
7   from commercially available products such as R-Gene™ or
8   any other source of pharmaceutical grade L-arginine, and
9   the nitroglycerin can be obtained from a variety of
10   delivery systems well known in the art for nitroglycerine
11   alone, for example: lingual aerosols such as
12   Nitrolingual™ spray (.4 mg / metered dose from Poulenc
13   Rorer); transdermal systems such as Minitran™ (.6 mg/hour
14   from 3M); topical ointments such as Nitro-Bid™ Ointment
15   (2% from Marion Merrell Dow as well as tablet and patch
16   form (currently using commercial patch product called
17   Tridil™ from Du Pont). This list is not all inclusive,
18   but is merely meant as a representation of the variety of
19   nitroglycerin delivery systems which could be easily
20   modified to be a delivery system for the combination of
21   L-arginine and nitroglycerin. All that is required is
22   compatible systems for the simultaneous delivery of
23   nitroglycerine and L-arginine. Such a selection of
24   delivery systems and commercial starting materials does
25   not depart from the scope and spirit of the present
26   invention. Hence, the true scope of the invention is
27   only to be defined by the claims appended hereto.

1   WHAT IS CLAIMED IS:

1   1.   A method for preventing or treating a disease
2   condition in a subject by vasodilation or vasorelaxation
3   comprising:
4   selecting a subject;
5   mixing a venous dilator and an arterial dilator;
6   administering to said subject a formulation
7   comprising said mixture;
8   obtaining periodic indicators of vasorelaxations for
9   the subject; and;
10   continuing administration of the formulation until a
11   desirable state of vasorelaxtion is obtained.

1   2.   The method of claim 1, wherein the formulation
2   is administered intravenously, buccal, intracoronary,
3   intramuscularly, topically, intranasally, rectally,
4   sublingually, orally, subcutaneously, or by patch.

1   3.   The method of claim 1, wherein said arterial
2   dilator is L-arginine.

1   4.   The method of claim 1, wherein said disease is
2   hypertension, hypertensive heart disease, coronary heart
3   disease, cardiovascular disease, cerebrovascular disease,
4   and renovascular ischemia.

1   5.   The method of claim 3, wherein said venous
2   dilator is an exogenous source of nitric oxide.

1   6.   The method of claim 5, wherein said exogenous
2   source of nitric oxide is nitroglycerin.

1   7.   The method of claim 5, wherein said exogenous
2   source of nitric oxide is selected from the group
3   consisting of sodium nitroprusside, nitrate esters,
4   isoamylynitrite, SIN-1, cysteine, dithiothreitol, N-

5   acetylcysteine, mercaptosuccinic acid, thiosalicylic
6   acid, and methylthiosalicylic acid.

1   8.   The method of claim 6, wherein L-arginine and
2   nitroglycerin are administered at a therapuetic
3   concentration.

1   9.   The method of claim 8, wherein the therapuetic
2   concentration of L-arginine is from 7.5% to about 30% w/v
3   (g/ml).

1   10.   The method of claim 8, wherein the therapuetic
2   concentration of L-arginine is from 10% to about 15% w/v
3   (g/ml).

1   11.   The method of claim 8, wherein the therapuetic
2   concentration of L-arginine is 10% w/v (g/ml).

1   12.   The method of claim 8, wherein the therapeutic
2   concentration of nitroglycerin is from about .2
3   μg/kg/minute to about 5 μg/kg/minute.

1   13.   The method of claim 8, wherein the therapeutic
2   concentration of nitroglycerin is from about .5
3   μg/kg/minute to about 3 μg/kg/minute.

1   14.   The method of claim 8, wherein the therapeutic
2   concentration of nitroglycerin is from about .75
3   μg/kg/minute to about 2 μg/kg/minute.

1   15.   The method of claim 8, wherein the therapeutic
2   concentration of nitroglycerin is about 1 μg/kg/minute.

1   16.   The method of claim 8, wherein the pH is
2   maintained within the range of 6 to 8.0.

1   17.   The method of claim 8, wherein the pH is
2   maintained within the range of 7 to 7.4.

1   18.   A therapeutic mixture comprising a mixture of
2   L-arginine and an agonist of nitric oxide synthase.

1   19.   The therapeutic mixture of claim 18, wherein
2   the agonist is nitroglycerin.

1   20.   The therapeutic mixture of claim 18, wherein
2   the agonist is further comprised of a receptor mediated
3   agonist selected from the group consisting of:
4   acetylcholine, substance P, Histamine, arginine
5   vasopressin, bradykinin, Adenosine Triphosphate,
6   Prostaglandin F_{2a}, Oxytocin, Endothelium B, and the
7   calcium ionophore A23187.

1   21.   A method of stimulating nitric oxide synthase
2   to produce nitric oxide, said method comprising:
3   mixing L-arginine and an agonist of nitric oxide
4   synthase;
5   administering the mixture to a subject having a
6   nitric oxide synthase receptor site; and;
7   stimulating said nitric oxide synthase to a
8   desirable level.

1   22.   The method of claim 21, wherein said L-arginine
2   is in excess to said agonist.

1   23.   The method of claim 21, wherein the agonist is
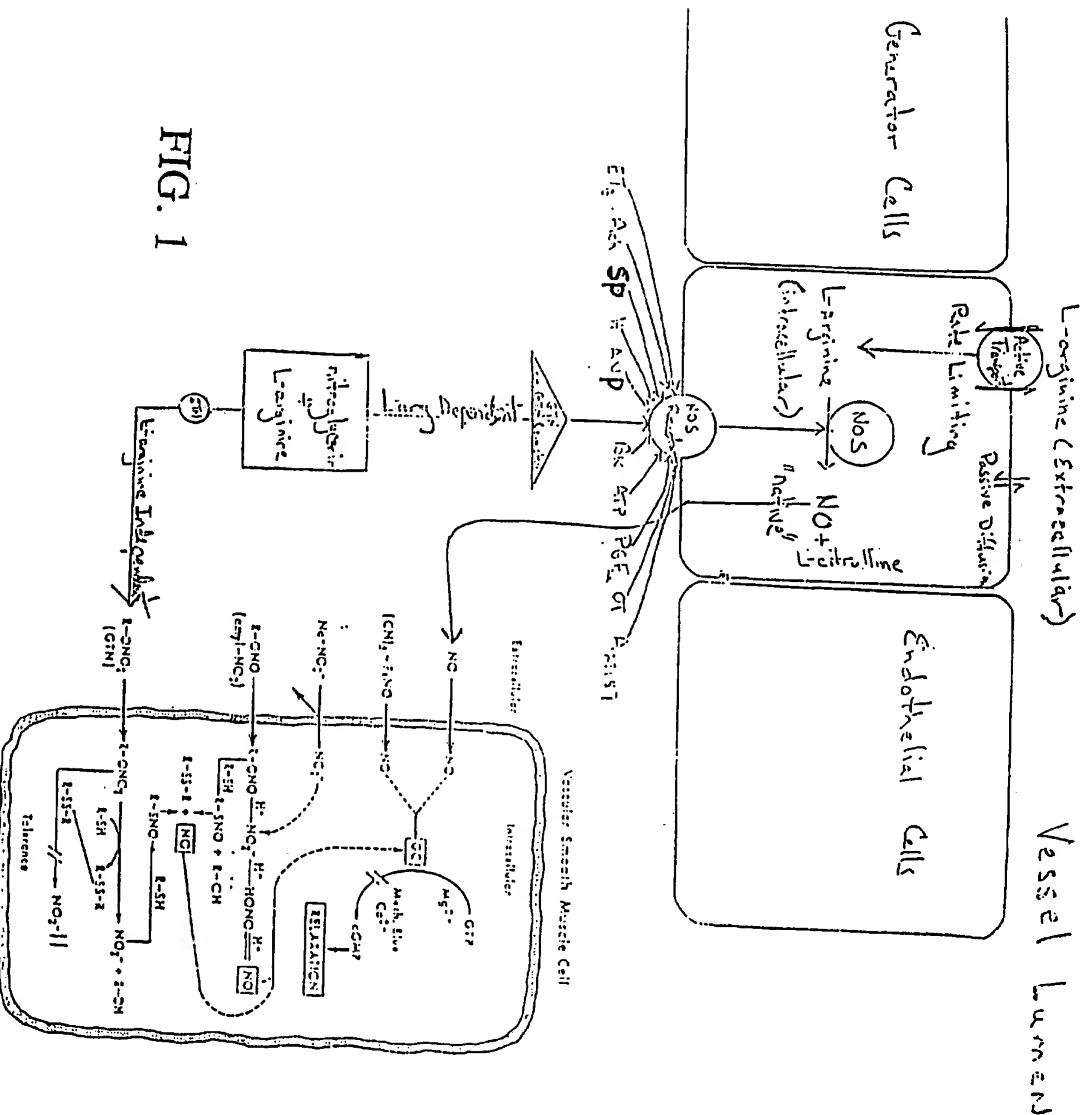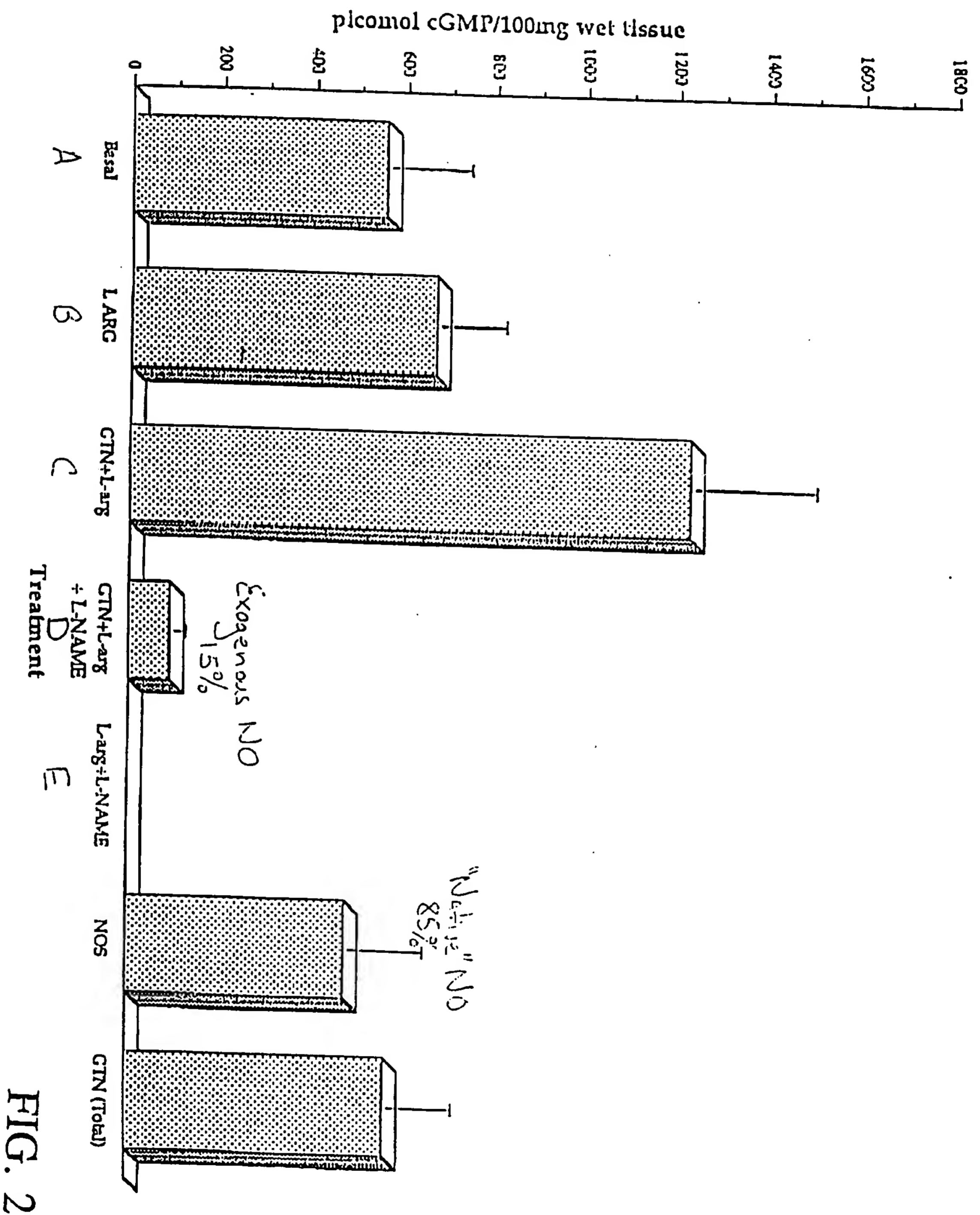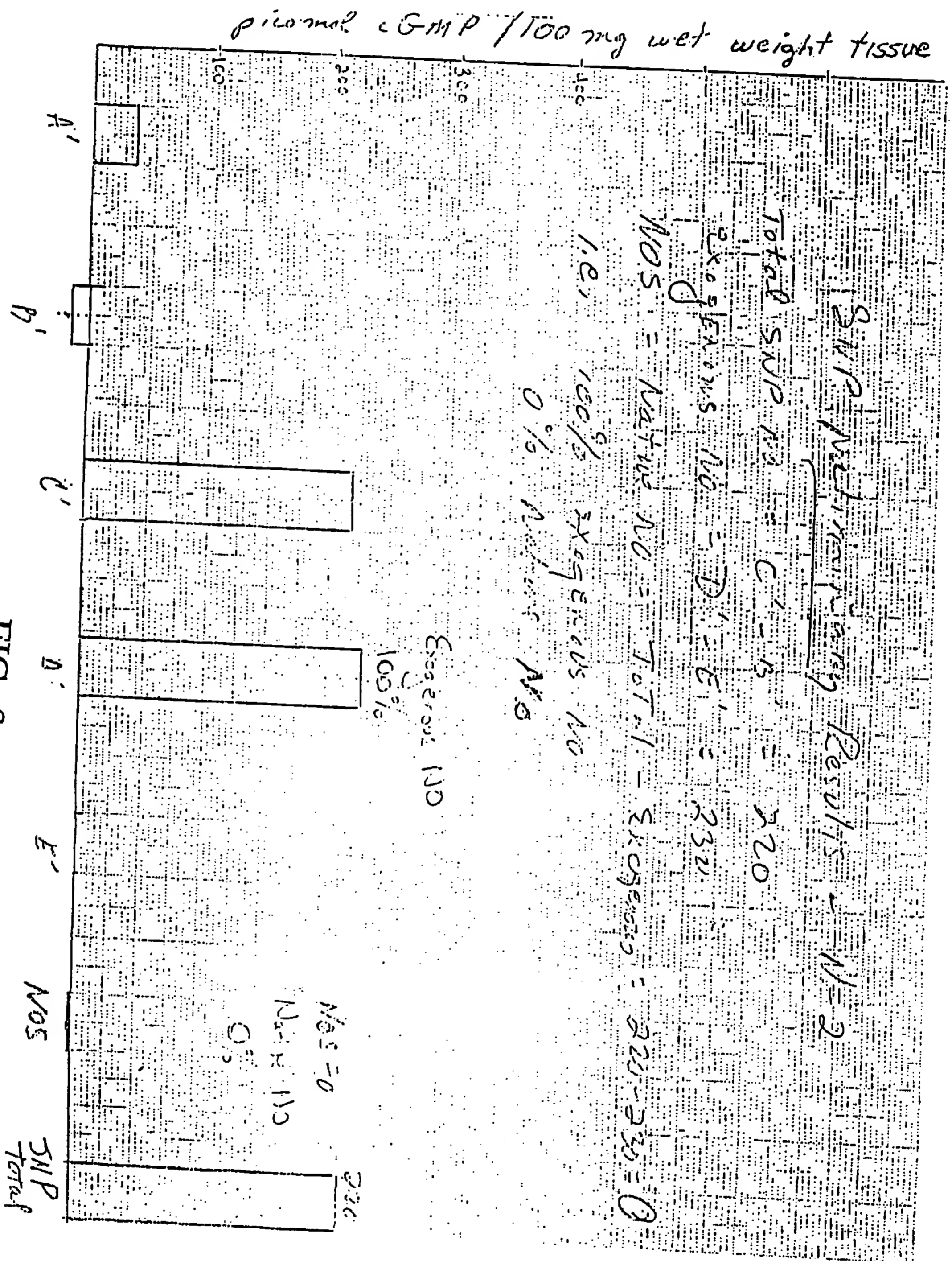2   nitroglycerin.

FIG. 1

Vessel Lumen
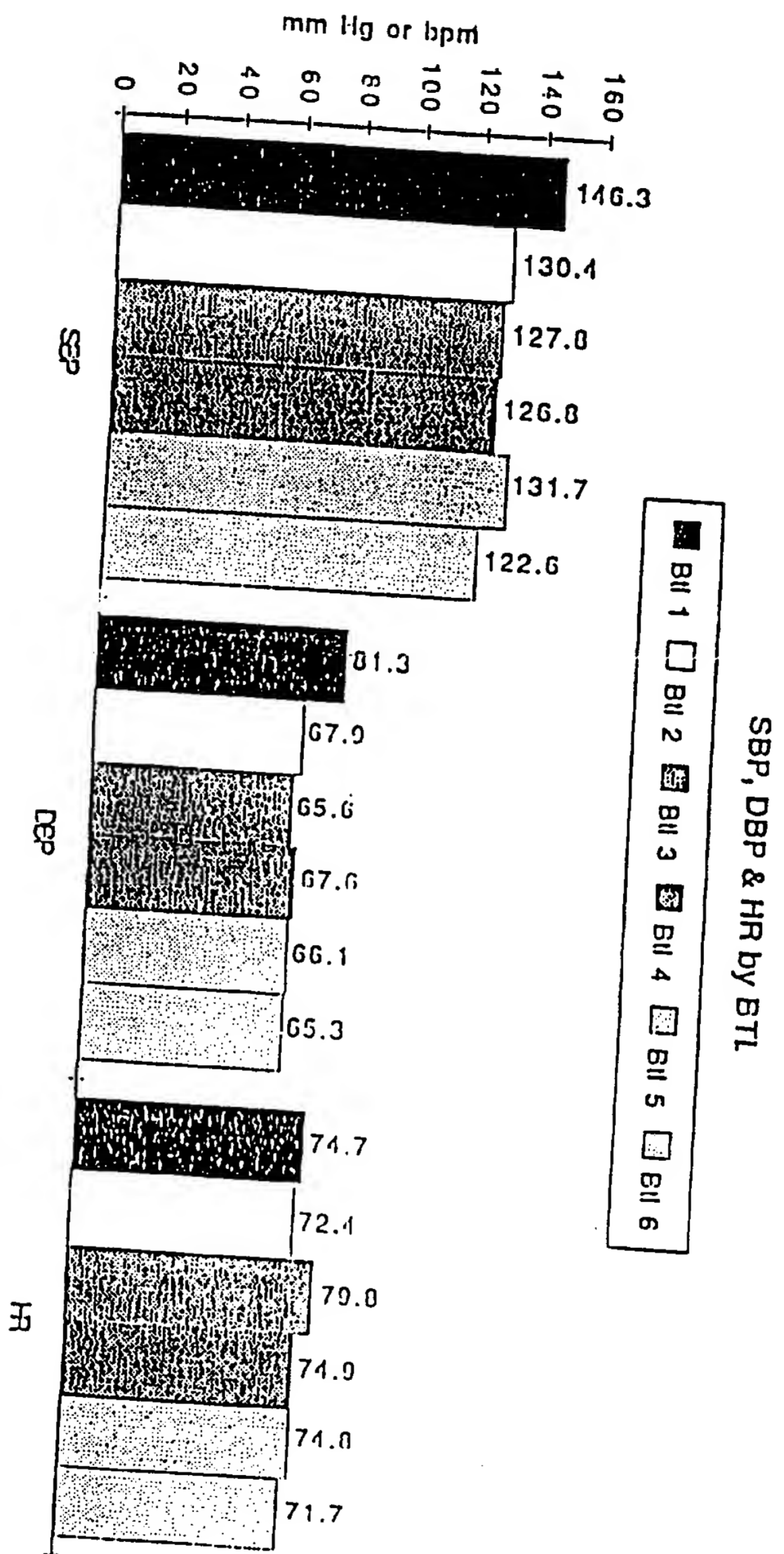
Generator Cells

Endothelial Cells

Vessel Wall

picomol cGMP/100mg wet tissue

FIG. 2

Exogenous NO
15%

"Native" NO
85%

picomol cGMP/100 mg wet weight tissue

Summarizing Results N=2

Total SUP n=13   C=R   D= R20

 D exogenous n=   D'= E'=.23v

NOS = Native no

i.e. 100% exogenous no

0% Native No

C = R20

Total - Exogenous = 220-2w=0

Exogenous (1J) 100%

NOS = 0
(1) N= 1(1)
0%

SUP Total

FIG. 3

FIG. 4

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 37/12
US CL :514/565

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/565

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN CAS FILE CA; FILE MEDLINE; FILE BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cardiovasc. Drug Therapy, vol. 8, no. 4, issued August 1994, Bassenge, "Coronary vasomotor responses: Role of endothelium and nitrovasodilators," pages 601-610, see Medline Abstract 95151614.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 JANUARY 1996	08 FEB 1996

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PAUL KILLOS
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